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by Preventing HIF-1 Activation

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Hypoxia inducible factor-1 (HIF-1) is a transcription factor that governs cellular responses to reduced O<sub>2</sub> availability by mediating crucial homeostatic processes. The degradation of HIF-1 $\alpha$  subunit is redox regulated. Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme that can modulate cellular redox environment. Here we show that MnSOD suppresses hypoxic accumulation of HIF-1 $\alpha$  protein. This suppression is biphasic depending on MnSOD activity. At low levels of MnSOD activity, HIF-1 $\alpha$  protein accumulates under hypoxic conditions in human breast carcinoma MCF-7 cells. At moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells), these accumulations are blocked. However, at higher levels of MnSOD activity (>6-fold increase), accumulation of HIF-1 $\alpha$  protein is again observed and the HIF-1 $\alpha$  protein level increases with increasing MnSOD activity. This biphasic modulation can be observed under both 1% O<sub>2</sub> and 4% O<sub>2</sub>. Hypoxic induction of vascular endothelial growth factor (VEGF), a known HIF-1 targeted gene, is also suppressed by elevated MnSOD activity and its expression levels reflected the protein levels of HIF-1 $\alpha$ . These observations demonstrate that HIF-1 $\alpha$  accumulation is modulated not only by the levels of dioxygen but also the antioxidant enzyme MnSOD.

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## Introduction

Tumor cells that have adapted to hypoxic conditions are thought to play critical roles in tumor progression (1). Among the first responses at the onset of hypoxia is an increase in the protein levels of hypoxia-inducible factor-1 (HIF-1) (2). HIF-1 responds to reduced O<sub>2</sub> availability by mediating crucial homeostatic processes such as angiogenesis, glycolysis, and erythropoiesis (3). As a transcription factor, HIF-1 has more than 40 target genes and the known number continues to increase. Vascular endothelial cell growth factor (VEGF) is one of the HIF-1 downstream genes (4). It is a powerful mitogenic cytokine specific for endothelial cells; its production triggers the angiogenic cascade and tumor neovascularization process (5). VEGF controls not only the onset, but also the extent and duration of these processes. Hypoxia, which is a common characteristic of solid tumors, is a stimulus leading to the induction of VEGF *via* the regulation of HIF-1 (6-8). Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are known to serve as signal transducers (9-12). Manganese superoxide dismutase (MnSOD) is a major antioxidant enzyme that is located in the mitochondrial matrix. It has been shown that the malignant phenotype is suppressed when MnSOD activity is elevated in tumor cells (13-15). It has been implicated as a tumor suppressor and as a metastasis suppressor in some tumor cell lines (16-18). MnSOD modulates the cellular redox environment by converting superoxide radical (O<sub>2</sub><sup>•-</sup>) to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and dioxygen. Therefore, MnSOD may affect the expression of redox-sensitive genes, including HIF-1.

## Body

The objective of this research is to examine the relationship of MnSOD activity to breast tumor angiogenesis. Three Specific Aims were initially proposed:

- (1) Determine if MnSOD overexpression alters HIF-1 activity.
- (2) Determine if MnSOD overexpression alters VEGF expression in *H-ras* transformed human breast cancer cells.
- (3) Determine if MnSOD overexpression alters endothelial cell proliferation and migration.

Specific Aims I and II were accomplished this year. MnSOD overexpression was found to suppress HIF-1 $\alpha$  protein accumulation with hypoxia exposure. Moreover, the induction of one of the HIF-1 targeted genes, VEGF, was suppressed by MnSOD-overexpression in a similar pattern as that of HIF-1 $\alpha$  protein. However, instead of using *H-ras* transformed cells that upon reflection we thought would too specific for the purpose of the study, we used human the breast cancer cell line MCF-7 wild type to test the suppressive effect of MnSOD on HIF-1 and VEGF. The five major tasks in the SOW are listed below and progress on each is summarized.

*Task 1: Development of breast cancer cells with altered MnSOD expression.*

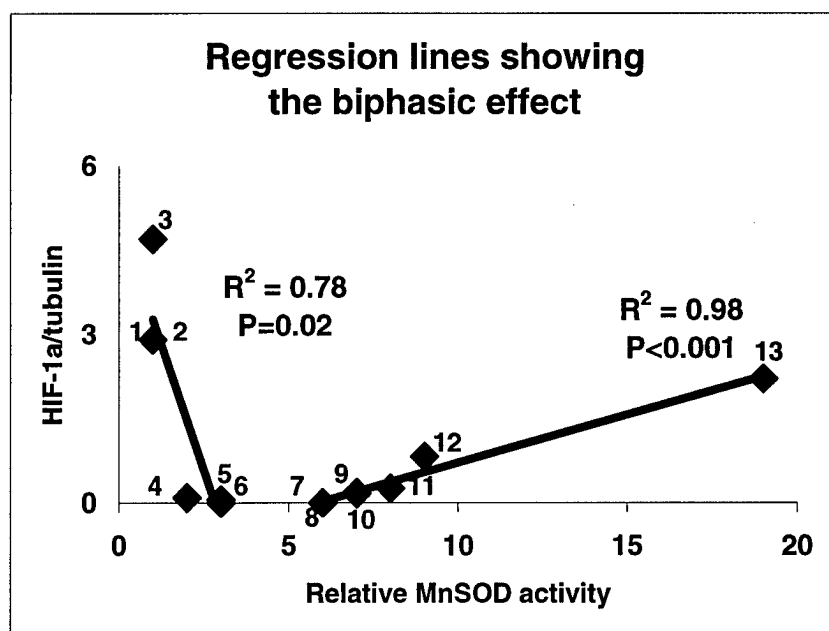
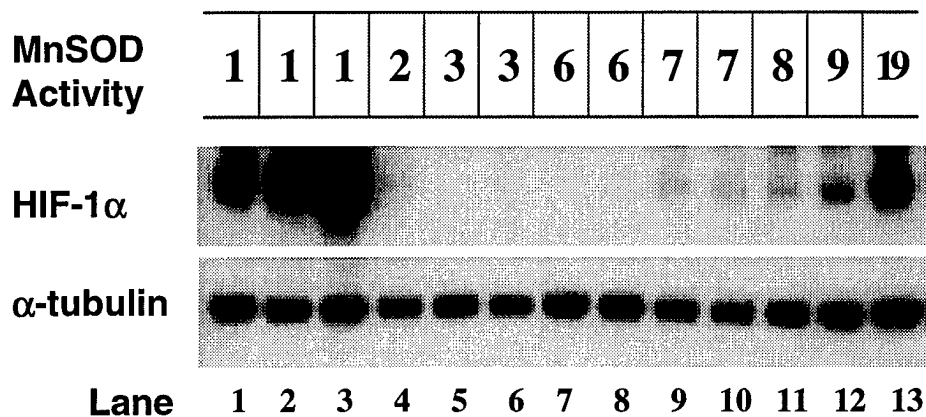
### ----Completed

During the first year of the research program (2002-2003), human breast cancer cells with altered MnSOD expression was developed as proposed in Task 1. I was lucky in that a large set of MCF-7 cells that have a wide range of MnSOD activity as a result of stable plasmid transfection became available. I characterized the antioxidant profile of these cells so as to make an informed choice of which subset of these cells would be used for this study. In addition I adapted protocols to use adenoviral induction to elevate MnSOD activity in MCF-7 cells. The protocols needed to produce a range of MnSOD activity in MCF-7 cells were developed and protein expression and activities levels were verified.

*Task 2: Determine if level of MnSOD activity alters HIF-1 activation in human breast cells.*

### ----Completed

We have shown that MnSOD suppressed hypoxic accumulation of HIF-1 $\alpha$  protein in human breast carcinoma MCF-7 cells. This suppression was biphasic depending on MnSOD activity. At low levels of MnSOD activity, HIF-1 $\alpha$  protein accumulated under hypoxic conditions. At moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells), these accumulations were blocked. However, at higher levels of MnSOD activity (>6-fold increase), accumulation of HIF-1 $\alpha$  protein was again observed. This biphasic modulation can be observed under both 1% O<sub>2</sub> and 4% O<sub>2</sub>. (The Figure below shows the results at 1% oxygen.)



**Figure. Plasmid transfection of MnSOD suppressed HIF-1 $\alpha$  protein levels at 1% O<sub>2</sub>.**

Top: Western blot showed HIF-1 $\alpha$  protein expression in 13 different MnSOD stably transfected clones of MCF-7 cells after 4 h of growth in 1% O<sub>2</sub>. The first 3 lanes were samples from: MCF-7 parent cells, Neo vector control and a clone with same activity as parental cells, respectively. The remaining 10 lanes were samples from clones with increasing MnSOD activity. The top row of numbers showed the relative MnSOD activity compared to the parental MCF-7 cells. Similar results have been obtained in a repeat experiment.

Bottom: Regression analysis shows a biphasic effect. HIF-1 $\alpha$  protein levels were quantified by densitometry and normalized to  $\alpha$ -tubulin levels. Thirteen points are shown, some overlay. The numbers next to points correspond to the lane number in Figure IV-3a.

We did parallel experiments at 4% oxygen and observed similar trends, however as might be expected at these higher levels of oxygen, HIF-1 $\alpha$  protein levels were decreased overall compared to 1% oxygen.

In addition, we demonstrated that HIF-1 $\alpha$  was regulated post-translationally in the above experimental settings as HIF-1 $\alpha$  mRNA was not changed significantly with hypoxia exposure compared to the changes of protein levels. These observations demonstrate that HIF-1 $\alpha$  accumulation can be modulated by the antioxidant enzyme MnSOD.

- Use gel shift and supershift to measure the DNA binding activity of HIF-1 transcriptional complex.

----Since the expression of one of the HIF-1 downstream gene, VEGF, was studied to verify the activity of HIF-1 under hypoxia, this subtask was substituted by the gene expression study of VEGF.

*Task 3: Determine if level of MnSOD activity alters the level of VEGF expression or the level of VEGF 121 isoform in human breast cells.*

----**Completed**

- Generate a riboprobe for VEGF<sub>121</sub>.

---- VEGF primer used for RT-PCR was designed and tested.

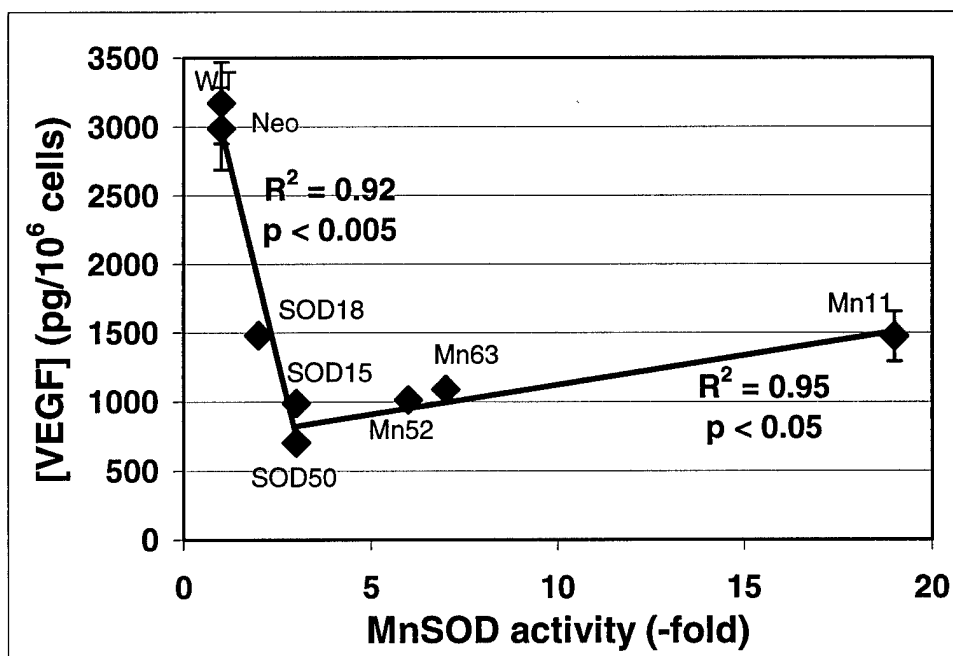
- Subject cells to conditions known to activate VEGF expression (hypoxia).

----Routine work.

- Use western and northern blotting to measure protein and mRNA of VEGF.

----Both VEGF mRNA and protein levels were suppressed in a similar pattern as that of HIF-1 $\alpha$  protein with the overexpression of MnSOD. RT-PCR was used to measure qualitatively mRNA levels of VEGF. VEGF Elisa assay was used to measure VEGF protein levels.

Hypoxic induction of vascular endothelial growth factor (VEGF), a known HIF-1 target gene, was also suppressed by elevated MnSOD activity and its expression levels reflected HIF-1 $\alpha$  protein levels.



**Figure.** MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. VEGF protein was measured after 12 h of exposure to hypoxia (1% O<sub>2</sub>) in 8 distinct MCF-7 clones with various MnSOD activities. VEGF protein secretion was suppressed with increased MnSOD activity. Regression analysis shows a biphasic effect parallel to the observations with HIF-1 $\alpha$ .

These observations demonstrate that HIF-1 $\alpha$  accumulation can be modulated by the antioxidant enzyme MnSOD AND that this results in the parallel control over the expression of a key gene controlled by HIF-1 $\alpha$ .

*Task 4: Determine if level of MnSOD activity in breast cancer cells alters proliferation and migration of nearby endothelial cells.*

This task has not been started. We submitted our results from tasks 1-3 for publication and the reviewers asked that we address the molecular mechanism behind our observations. Because of the importance of this question we devoted our energies to the question: *Is superoxide or hydrogen peroxide involved in the observations summarized above?* Because MnSOD controls the steady-state level of superoxide and produces hydrogen peroxide as a result of its enzymatic action, experiments were designed to test a possible role of hydrogen peroxide in our observations. To address this question we devised methods to alter the ability of the cells to remove hydrogen peroxide. We inhibited catalase activity and we increased catalase activity (verified using enzyme assays as well as actual measurements of hydrogen peroxide production) and observed that this modulated the expression of HIF-1 $\alpha$  only in the Mn11 clone, i.e. the clone with the highest level of MnSOD and the highest level of hydrogen peroxide production. To provide another test, we used adenovirus transduction of glutathione peroxidase (GPx-1), another peroxide-removing enzyme and observed exactly parallel results, i.e. only the Mn11 clone was affected. These data suggest that hydrogen peroxide is only a minor player in our observations and that superoxide is the key chemical species responsible for our observations.

As another approach to test the possible role of superoxide in the activation of HIF-1 $\alpha$ , we did pilot experiments of altering copper-zinc superoxide (CuZnSOD), the cytosolic SOD. It too modulated HIF-1 $\alpha$  protein expression. We are currently doing experiment in which which try other approaches to alter the steady-state level of superoxide in cells and determine the effect on the modulation of HIF-1 $\alpha$ .

*Task 5: Prepare manuscripts and Ph.D. thesis.*

A manuscript has been submitted to Onogene, see attached. It has received a favorable review and we are currently revising it.

I am preparing a PhD thesis. My goal is to submit it for Summer 2004 graduation. I have defended the thesis and currently working on revisions.

## Key Research Accomplishments

- Establishment of special protocols for HIF-1 $\alpha$  protein western blotting.  
Specified in the Materials & Methods section of the attached manuscript: Protein harvest for HIF-1 $\alpha$  western blot & HIF-1 $\alpha$  western blot.
- Original findings on the regulatory effect of MnSOD on hypoxic accumulation of HIF-1 $\alpha$ .  
We found that elevated MnSOD activity suppresses the hypoxic accumulation of HIF-1 $\alpha$  protein. The suppression of MnSOD on HIF-1 $\alpha$  protein accumulation was biphasic depending on MnSOD activity as summarized in the Body. This biphasic modulation can be observed under both 1% O<sub>2</sub> and 4% O<sub>2</sub> (Figure 1, 2 & 3).
- Findings on the regulatory effect of MnSOD on hypoxic induction of VEGF.  
Hypoxic induction of VEGF was also suppressed by elevated MnSOD activity and its expression levels reflected the protein levels of HIF-1 $\alpha$ . (Figures 4 & 5 for VEGF mRNA levels and Figures 6 & 7 for VEGF protein levels in the appendix).
- Findings on the molecular mechanism by which MnSOD affects HIF-1 $\alpha$  VEGF. We have determined that hydrogen peroxide appears to be a minor player in this effect and all indirect evidence points to a key role for superoxide.

## Reportable Outcomes

### Abstracts/Presentations:

Wang MV, Zhang HJ, Kirk JS, et al. HIF-1 induction under hypoxia is modulated by MnSOD *FREE RADICAL BIOLOGY AND MEDICINE* 33: 424 Suppl. 2 2002

**M. Wang**, H.J. Zhang, J.S. Kirk, L.W. Oberley, G.R. Buettner. (2002) Modulating HIF: Potential targets for cancer therapy. Poster at 2<sup>nd</sup> International conference on prostate cancer research, Iowa City, IA, October 12-15.

### Fellowship based on this award:

Graduate Incentive Fellowship, 2002, The University of Iowa.



## Conclusions

The study is the first to demonstrate that not only dioxygen, but also the antioxidant enzyme MnSOD, which modulates the levels of superoxide and hydrogen peroxide, can determine the level of HIF-1 $\alpha$ , and consequently the expression of VEGF, an essential factor for the growth of new blood vessels. This understanding of the control of HIF-1 $\alpha$  and subsequent induction of VEGF points to new interventions that can enhance recovery from stroke, heart attack, trauma as well as suggests new avenues for improved cancer therapy.

To be able to apply the presented research results for clinical studies, a better understanding of the regulatory mechanism is necessary. Therefore, we proposed to further pursue the molecular mechanisms controlling the biphasic suppressive effect of MnSOD on HIF-1 $\alpha$  protein. Since one of the products of MnSOD enzymatic activity is hydrogen peroxide, which can diffuse across membranes freely, we examined the potential role of hydrogen peroxide as a key mediator of the suppressive effect of MnSOD. To address this question, the role of peroxide removing enzymes (catalase and glutathione peroxidase) was tested. We found that they produced only a minor effect on HIF-1 $\alpha$  activation. Indirect evidence points to superoxide as a key species in the regulation of HIF-1 $\alpha$ . Further experiments are in progress to better test the role of superoxide in HIF-1 $\alpha$  activation.

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Appendix

Wang, M

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**Manganese superoxide dismutase suppresses hypoxic induction of  
hypoxia inducible factor-1 $\alpha$  and vascular endothelial growth factor**

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## Abstract

Hypoxia inducible factor-1 (HIF-1) is a transcription factor that governs cellular responses to reduced O<sub>2</sub> availability by mediating crucial homeostatic processes. HIF-1 is composed of a HIF-1 $\alpha$  subunit and a HIF-1 $\beta$  subunit. HIF-1 $\alpha$  is degraded *via* enzyme-dependent hydroxylation of prolines of HIF-1 $\alpha$  in the presence of molecular oxygen, Fe<sup>2+</sup>,  $\alpha$ -ketoglutarate, and ascorbate. These cofactors contribute to the redox environment of cells. The antioxidant enzyme manganese superoxide dismutase (MnSOD) also modulates the cellular redox environment. Here we show that MnSOD suppressed hypoxic accumulation of HIF-1 $\alpha$  protein in human breast carcinoma MCF-7 cells. This suppression was biphasic depending on MnSOD activity. At low levels of MnSOD activity, HIF-1 $\alpha$  protein accumulated under hypoxic conditions. At moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells), these accumulations were blocked. However, at higher levels of MnSOD activity (>6-fold increase), accumulation of HIF-1 $\alpha$  protein was again observed. This biphasic modulation can be observed under both 1% O<sub>2</sub> and 4% O<sub>2</sub>. Hypoxic induction of vascular endothelial growth factor (VEGF), a known HIF-1 target gene, was also suppressed by elevated MnSOD activity and its expression levels reflected HIF-1 $\alpha$  protein levels. These observations demonstrated that HIF-1 $\alpha$  accumulation could be modulated by the antioxidant enzyme MnSOD.

## Introduction

Hypoxia describes a low oxygen concentration environment that can be caused by stroke, coronary artery disease, trauma, or high rate of cell proliferation such as encountered in solid tumors (Dewhirst et al., 1989). Because the proliferation rate of tumor cells is usually faster than that of normal endothelial cells that form microvessels into tumors, tumor microvessels do not function as effectively and efficiently as those in normal tissues, leading to the widespread hypoxia in solid tumors (Shah-Yukich & Nelson, 1988).

Tumor cells that have adapted to hypoxic conditions are thought to play critical roles in tumor progression (Hockel et al., 1996). Among the first responses at the onset of hypoxia is an increase in the protein levels of hypoxia-inducible factor-1 (HIF-1) (Wang & Semenza, 1993b). HIF-1 responds to reduced O<sub>2</sub> availability by mediating crucial homeostatic processes such as angiogenesis, glycolysis, and erythropoiesis (Semenza, 2000). As a transcription factor, HIF-1 has more than 40 target genes and the known number continues to increase. Vascular endothelial cell growth factor (VEGF) is one of HIF-1 downstream genes (Liu et al., 1995). It is a powerful mitogenic cytokine specific for endothelial cells; its production triggers the angiogenic cascade and tumor neovascularization processes (Nagy et al., 2002). VEGF controls not only the onset, but also the extent and duration of these processes. Hypoxia, which is a common characteristic of solid tumors, is a stimulus leading to the induction of VEGF *via* the regulation of HIF-1 (Kim et al., 2003; Pugh & Ratcliffe, 2003a; Semenza, 2001b).

HIF-1 is a heterodimer consisting of two subunits, HIF-1 $\alpha$  and HIF-1 $\beta$  (Wang & Semenza, 1993a). When oxygen is sufficient, HIF-1 $\alpha$  is constantly being made and then degraded by the ubiquitin-proteasome pathway *via* von Hippel-Lindau (VHL) tumor suppressor (Maxwell et al., 1999). VHL protein binds to HIF-1 $\alpha$ , causing ubiquitinylation of this protein, which is then degraded by the 26S proteasome (Salceda & Caro, 1997). The binding of VHL to HIF-1 $\alpha$  requires

hydroxylation of proline residues inside the oxygen-dependent degradation domain (ODD) of HIF-1 $\alpha$  (Bruick & McKnight, 2001). Proline<sup>402</sup> and proline<sup>564</sup> are hydroxylated by HIF-prolyl hydroxylase, which needs the presence of several cofactors to gain full activity. These cofactors are: molecular oxygen, Fe<sup>2+</sup>,  $\alpha$ -ketoglutarate, and ascorbate (Jaakkola et al., 2001; Kondo & Kaelin, 2001; Pugh & Ratcliffe, 2003b; Semenza, 2001a). During hydroxylation, one atom of the O<sub>2</sub> molecule is incorporated into succinate upon decarboxylation of  $\alpha$ -ketoglutarate and the other into a hydroxyl group on the proline residue. The Fe<sup>2+</sup> is located inside the active site of HIF-prolyl hydroxylase (McNeill et al., 2002).

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are known to serve as signal transducers (Nakamura et al., 1997; Powis et al., 1997; Schafer & Buettner, 2001; Suzuki et al., 1997). It has been shown that ROS can participate in the hypoxia signal transduction pathway that mediates the stabilization of HIF-1 $\alpha$  (Chandel et al., 2000; Park et al., 2003; Schroedl et al., 2002). Manganese superoxide dismutase (MnSOD) is a primary antioxidant enzyme that is located in the mitochondrial matrix. It has been shown that the malignant phenotype is suppressed when MnSOD activity is elevated in tumor cells (Li et al., 1998a; Li et al., 1998b; Zhang et al., 1999). MnSOD has been implicated as a tumor suppressor and as a metastasis suppressor in some tumor cell lines (Bravard et al., 1992; Oberley & Buettner, 1979; Oberley & Oberley, 1988). It modulates the cellular redox environment by converting superoxide radical (O<sub>2</sub><sup>•-</sup>) to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and dioxygen. Therefore, MnSOD may affect the expression of redox-sensitive genes, including HIF-1. Here we are the first to show that MnSOD suppressed the hypoxic accumulation of HIF-1 $\alpha$  in human breast carcinoma MCF-7 cells. The hypoxic induction of VEGF mRNA and protein were also suppressed by MnSOD.

## Results

### Increased MnSOD activity suppressed hypoxic accumulation of HIF-1 $\alpha$ protein in cells exposed to 1% O<sub>2</sub>.

To test the role of elevated MnSOD activity on hypoxic accumulation of HIF-1 $\alpha$  protein, twelve previously characterized (Zhang et al., 1999) stably transfected MCF-7 clones with different MnSOD activities as well as parental cells were used. These 13 different cell lines were exposed to 1% O<sub>2</sub> for 4 h. This condition did not change MnSOD activity (data not shown). Immediately after hypoxia, protein was harvested and HIF-1 $\alpha$  protein levels were analyzed by western blotting (**Figure 1a**). The results showed that at low levels of MnSOD activity (parental cells and clones with the same activity), HIF-1 $\alpha$  protein accumulated. In clones where MnSOD activity was moderately increased (2- to 6-fold relative to that of the parent cells), HIF-1 $\alpha$  protein levels were decreased. The levels of HIF-1 $\alpha$  protein decreased with increasing MnSOD activity until they were no longer detectable in clones with 3- and 6-fold increase in MnSOD activity. However, in clones where MnSOD activity was increased to a higher level (> 6-fold), HIF-1 $\alpha$  protein was again detected, and increased with increasing MnSOD activity. Regression analysis demonstrated a linear inverse correlation of HIF-1 $\alpha$  with low levels of MnSOD activity (1- to 3-fold), whereas at high levels of MnSOD (> 6-fold), a linear positive correlation was observed (**Figure 1b**).

**Figure 1**

In order to confirm and extend this observation with another technique besides the stable transfection approach, we further studied this effect in MCF-7 cells that were transduced with adenovirus-containing MnSOD cDNA. This transduction increased the enzymatic activity of MnSOD as determined by activity gel analyses; MnSOD activity increased with increasing MOI (multiplicity of infection, which is calculated from PFU, plaque forming units, **Figure 2a**). The

**Figure 2**

increase in SOD activity with increasing MOI was confirmed with a spectrophotometric enzymatic activity assay (data not shown). Following adenovirus transduction in 21% O<sub>2</sub>, cells were exposed to 1% O<sub>2</sub> for 4 h and samples were then collected. SOD activity (activity gel assay) and HIF-1 $\alpha$  protein levels (western blotting) were assessed. In MCF-7 parental cells, HIF-1 $\alpha$  protein accumulated after hypoxia exposure; increased MnSOD activity suppressed the accumulation of HIF-1 $\alpha$  protein under 1% O<sub>2</sub> (**Figure 2b**). HIF-1 $\alpha$  protein levels dropped significantly starting at 5 MOI of adenoviral MnSOD and became undetectable at 50 MOI. HIF-1 $\alpha$  protein was again detectable at 75 MOI and increased with increasing MOI thereafter, but not reaching the level found in parental cells. This is consistent with what we observed with MCF-7 clones that were stably transfected with MnSOD cDNA, **Figure 1**. In both plasmid transfection and adenovirus transduction experiments, HIF-1 $\alpha$  protein was undetectable in cells grown at 21% O<sub>2</sub> (data not shown).

**Increased MnSOD activity suppressed hypoxia accumulation of HIF-1 $\alpha$  protein in cells exposed to 4% O<sub>2</sub>.**

The oxygen concentration inside a large solid tumor is heterogeneous. Some regions have very low pO<sub>2</sub> values while others have pO<sub>2</sub> values near to that of normal tissues (Collingridge et al., 1997; Moulder & Martin, 1984). Because MnSOD suppressed HIF-1 $\alpha$  protein accumulation in MCF-7 cells exposed to 1% O<sub>2</sub>, both with adenoviral transduction and plasmid transfection, we further investigated if this suppressive effect existed when cells were exposed to 4% O<sub>2</sub>, a value typical of normal tissue oxygen concentration but still lower than the 21% O<sub>2</sub> usually used for most cell culture conditions. Using the same stable MnSOD-overexpressing clones described above, we found that HIF-1 $\alpha$  protein began to decrease in clones that expressed a 2-fold increase in MnSOD activity and remained suppressed in clones that had up to a 6-fold increase in MnSOD activity. However, when MnSOD activity was increased higher than 8-fold, HIF-1 $\alpha$  levels were



again increased (**Figure 3a**). In order to examine this with another method, MCF-7 cells transduced at 21% O<sub>2</sub> with increasing MOI of adenoviral MnSOD were then exposed to 4% O<sub>2</sub>. As MnSOD activity increased from that of parental MCF-7 cells, HIF-1 $\alpha$  levels decreased, reaching a minimum at 20 and 50 MOI (**Figure 3b**). At 75 MOI, HIF-1 $\alpha$  levels increased and continued to increase with increasing MOI, but again not reaching the level of parental cells. The results observed at 4% O<sub>2</sub> parallel those of 1% O<sub>2</sub>, suggesting that the suppressive effect of MnSOD on HIF-1 $\alpha$  is common at both oxygen concentrations.

#### **Hypoxia (1% O<sub>2</sub>) induced VEGF mRNA expression in a time-dependent manner in MCF-7 cells.**

Angiogenesis is a key step that not only supports tumor growth with nutrients and oxygen, but also provides a ready route for cells to escape the primary site, leading to metastases (Brown et al., 1997). Vascular endothelial growth factor is a potent angiogenic mitogen that mediates the angiogenesis process (Dvorak et al., 1995a; Nagy et al., 2002). Exposure to hypoxia stimulates the production of VEGF, mainly through the induction of the transcription factor HIF-1 (Kim et al., 2003). With the observation that MnSOD suppressed hypoxic accumulation of HIF-1 $\alpha$  protein, we tested the effect of increasing MnSOD activity on hypoxic induction of VEGF in MCF-7 cells.

Cells were exposed to hypoxia (1% O<sub>2</sub>) for various times, then the cells were lysed and total RNA isolated to be used for semiquantitative RT-PCR. VEGF mRNA expression increased dramatically after cells were exposed to hypoxia for 6 h, compared to cells without hypoxia exposure. This induction continued as cells were exposed for longer times (**Figure 4**).

**Hypoxic induction of VEGF mRNA was suppressed by increased MnSOD enzymatic activity in cells exposed to 1% O<sub>2</sub>.**

As VEGF mRNA expression increased substantially with 6 h of hypoxia, we studied the effect of increased MnSOD activity on VEGF mRNA expression at this time. Because MnSOD modulated HIF-1 $\alpha$  and HIF-1 $\alpha$  accumulation leads to VEGF expression, we anticipated that MnSOD would modulate VEGF. Indeed, MnSOD transfection modulated VEGF expression with hypoxic exposure parallel to the observation with HIF-1 $\alpha$ . After cells were exposed to 6 h hypoxia, VEGF mRNA expression was induced in parental cells. In cells with intermediate levels of MnSOD, the induction was suppressed (**Figure 5a&b**). VEGF expression began to drop in the clone with 2-fold increase in MnSOD activity and reached the lowest levels in clones with 3-fold increase. In clones with higher MnSOD activity, VEGF expression was not suppressed as much as in clones with lower MnSOD activity (**Figure 5a&b**).

**Figure 5**

The suppressive effect was also observed with adenoviral induction of MnSOD. MCF-7 cells were transduced with 50 MOI of adenoviral MnSOD to increase MnSOD activity (HIF-1 $\alpha$  protein was suppressed most with this MOI, **Figure 2b**); cells were then exposed to hypoxia (1% O<sub>2</sub>) for 6 h, followed by total RNA isolation and semiquantitative RT-PCR for VEGF. Increased MnSOD activity suppressed the expression of VEGF mRNA by approximately 50%, compared to parental cells or cells transduced with *AdLacZ* (**Figure 5c**).

Glucose transporter-1 (GLUT-1) is another HIF-1 downstream gene. MnSOD transfection had only a minor effect on hypoxic induction of GLUT-1 mRNA (data not shown). However, MCF-7 cells have high endogenous GLUT-1 and hypoxia did not dramatically increase

its expression. Thus, increased MnSOD activity would be expected to show little effect on GLUT-1 compared to VEGF. Indeed only a small effect was observed (data not shown).

#### **MnSOD suppressed the secretion of VEGF protein with hypoxia stimulation (1% O<sub>2</sub>).**

As a mitogenic cytokine, VEGF protein is usually secreted outside the cells to stimulate the proliferation of endothelial cells (Dvorak et al., 1995b). To determine if VEGF protein secretion followed the trend of its mRNA expression, the pattern of VEGF protein secretion with hypoxia stimulation was examined. MCF-7 WT cells were exposed to hypoxia for various times and VEGF concentrations were measured. Hypoxia exposure increased the amount of VEGF protein (**Figure 6a**). The amount of VEGF protein increased rapidly from 8 until 12 h in the hypoxia-treated cells; after 12 h, VEGF levels kept increasing, but at a lower rate. VEGF protein also increased in non-hypoxic cells, but at a much slower rate. Moreover, most of the VEGF protein accumulated in the medium and only small amounts were found inside the cells (**Figure 6a**).

**Figure 6**

To determine the suppressive effect of elevated MnSOD activity on the secretion of VEGF protein, seven stably transfected MCF-7 clones with various MnSOD activity (described above) together with parental cells were exposed to hypoxia (1% O<sub>2</sub>) for 12 hours. Medium from these clones were then collected and VEGF concentrations were measured. Increased MnSOD activity significantly decreased VEGF protein levels (**Figure 6b**). The suppression of VEGF protein by MnSOD was biphasic, with the greatest suppression at low to medium MnSOD levels and less suppression at higher MnSOD levels, consistent with what we observed with HIF-1 $\alpha$  protein and VEGF mRNA levels.

**VEGF secretion over time is different in a clone with medium MnSOD activity compared to a clone with high MnSOD activity.**

To gain more insight into the influence of MnSOD on VEGF protein secretion, we tested VEGF protein levels from two clones after exposing them to hypoxia (1% O<sub>2</sub>) for various times. Clone SOD50 had a 3-fold increase in MnSOD activity compared to parental cells, whereas clone Mn11 had a 19-fold increase. These two clones had different responses after exposure to hypoxia for short times (**Figure 7**). A 3-fold increase in MnSOD resulted in a slower rate of VEGF accumulation than a 19-fold increase of MnSOD. Although both clones eventually reached the same plateau values, which were lower than that of parental cells, the initial different response of these two clones suggests that the mechanisms underlying the control of hypoxic accumulation of HIF-1 $\alpha$  with intermediate or high MnSOD activities may be different.

**Figure 7**

## **Discussion**

Hypoxia is a deficiency in oxygen (Ozaki et al., 1999), (Semenza et al., 1999). Hypoxia is widespread in solid tumors due to an inefficient vascular supply of oxygen. Hypoxia-inducible factor-1 (HIF-1) is an important transcription factor that is activated in conditions of decreased oxygen (Semenza et al., 1998). It mediates cell survival in hypoxia by promoting genes involved in glucose homeostasis, erythropoiesis, and angiogenesis (Semenza, 1999). While normal tissue and benign breast tumors do not exhibit increased HIF-1 activity, evidence for a graded increase in activity has been demonstrated in the progression from pre-neoplastic lesions to cancer metastases (Bos et al., 2001). Moreover, a positive correlation has been found between HIF-1 expression and vascularization in brain tumors (Zagzag et al., 2000).

As one of the HIF-1 downstream genes, VEGF is a pivotal mitogen that mediates endothelial cell proliferation and new vessel formation, a process known as angiogenesis. Hypoxic induction of VEGF is mediated by hypoxia inducible factor-1 (HIF-1) (Forsythe et al., 1996).

MnSOD is an important antioxidant enzyme involved in cancer cell growth. A change in the level of MnSOD enzyme activity will change the redox status of the cell and affect the expression of redox-sensitive genes and proteins. HIF-1 is among these redox-sensitive proteins (Chandel et al., 2000; Huang et al., 1996; Kelley & Parsons, 2001). It has been shown both *in vitro* and *in vivo* that tumor growth is suppressed with MnSOD overexpression (Li et al., 1998a; Li et al., 1998b; Zhang et al., 1999). MnSOD has been proposed as a tumor suppressor gene (Bravard et al., 1992; Oberley & Oberley, 1988). It catalyzes the reaction:  $2\text{O}_2^{\bullet-} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$ . A change in MnSOD activity will result in a change in the steady-state level of superoxide ( $\text{O}_2^{\bullet-}$ ). That a moderate increase in MnSOD decreased the accumulation of HIF-1 $\alpha$  suggests an involvement of superoxide. It has been observed that a moderate increase in nitric oxide ( $\text{NO}^{\bullet}$ ) decreased HIF-1 $\alpha$  accumulation (Mateo et al., 2003). Because  $\text{NO}^{\bullet}$  reacts rapidly with  $\text{O}_2^{\bullet-}$ , an increase in  $\text{NO}^{\bullet}$  will decrease the steady-state level of  $\text{O}_2^{\bullet-}$ , parallel to an increase in MnSOD activity. These observations suggest that superoxide could modulate HIF-1 $\alpha$  accumulation.

However, a product of MnSOD enzymatic activity, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), is a highly diffusible molecule that moves freely across cell membranes.  $\text{H}_2\text{O}_2$  also contributes to the redox environment of the cell (Oberley & Buettner, 1979). Therefore,  $\text{H}_2\text{O}_2$  may also be a link between MnSOD and HIF-1 $\alpha$ . Both superoxide and hydrogen peroxide could react with the iron ( $\text{Fe}^{2+}$ ) of HIF-prolyl hydroxylase, inhibiting its activity and leading to the accumulation of HIF-1 $\alpha$ .

We have shown here that MnSOD suppressed hypoxic accumulation of HIF-1 $\alpha$  protein, which led to lower levels of VEGF. Moderate levels of MnSOD activity (2- to 6-fold increase compared to parent cells) in MCF-7 cells abolished the hypoxic accumulation of HIF-1 $\alpha$  protein and the induction of VEGF. Surprisingly, higher levels of MnSOD allowed HIF-1 $\alpha$  protein accumulation, showing a biphasic modulation on HIF-1 $\alpha$  accumulation. Interestingly, in these

high MnSOD overexpressing cells, HIF-1 $\alpha$  never returned to values near those of the parent cells; in other words, HIF-1 $\alpha$  was still suppressed relative to parent cells and vector control. We hypothesize that elevation of MnSOD to moderate levels (physiological levels for most normal tissues) will lower the steady-state level of superoxide, which then lowers HIF-1 $\alpha$  levels. However, if MnSOD is increased above these levels, H<sub>2</sub>O<sub>2</sub> could be a modulator. An amount of peroxide is produced that is above the levels that the endogenous peroxide-removing capabilities of the cell can accommodate. Too much hydrogen peroxide then leads to inhibition of HIF-1 $\alpha$  degradation and HIF-1 $\alpha$  accumulates.

Another possibility for the effector of MnSOD is molecular oxygen. MnSOD produces both H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>. HIF-1 $\alpha$  degradation ensues upon the hydroxylation of prolines as accomplished by HIF prolyl hydroxylase. This specific prolyl hydroxylase requires 2-oxoglutarate, Fe<sup>2+</sup>, ascorbate, and molecular oxygen for enzymatic activity (Jaakkola et al., 2001; Kondo & Kaelin, 2001; Pugh & Ratcliffe, 2003b; Semenza, 2001a). All of these cofactors can be found in mitochondria as well as cytoplasm. Thus, the oxygen produced by MnSOD is also a possible effector for the MnSOD-suppressive effect we have observed.

Our results are consistent with what we have observed with tumor incidence in nude mice after MnSOD overexpression (Zhang et al., 1999). That is, the incidence of tumors decreases when MnSOD activity is increased to a moderate level (<6-fold). However, MnSOD does not further suppress the tumor formation when its activity is increased to a higher level (>6-fold), and indeed some of the tumor-suppression effect is lost (Zhang et al., 1999). This suggests that the *in vivo* tumor suppressive function of MnSOD may be achieved by the inhibition of HIF-1 protein accumulation. By controlling HIF-1 protein and thereby controlling the expression of HIF-1 downstream genes that are involved in tumor metabolism and growth, such as VEGF, MnSOD may control a switch for genes that allow tumor growth and progression. These results suggest

that MnSOD may inhibit tumor angiogenesis and that part of the tumor suppressive effect seen *in vivo* may be due to inhibition of angiogenesis.

In support of this proposal, Wheeler *et al.* have shown that adenovirus transduction of SOD3 (extracellular SOD or ecSOD) inhibits the growth of B16 melanoma cells in mice (Wheeler et al., 2003). Two weeks after implantation, B16 tumor size was 65% smaller in mice infected with *AdecSOD* in comparison with mice infected with *AdlacZ*. Tumors from *AdecSOD*-infected mice expressed less VEGF protein. Importantly, blood vessel density as assessed by two different ways was markedly reduced in tumors from *AdecSOD*-infected mice compared with controls. This study is consistent with our findings of the dramatic effect of SOD overexpression on HIF-1 $\alpha$  accumulation and VEGF expression.

## Materials and Methods

**Cell culture.** Human breast adenocarcinoma MCF-7 cells were routinely cultured in Eagles's MEM containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS, and incubated at 37°C with 95% air and 5% CO<sub>2</sub>. Medium was generally changed every 3-4 days.

**Hypoxic treatment.** Cells were seeded into 60 mm tissue culture dishes (Corning Scientific Products DIV, Acton, MA) and medium was replaced with fresh medium before hypoxic treatment. The dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was flushed with either 1% O<sub>2</sub> (1% O<sub>2</sub>, 5% CO<sub>2</sub> and balanced with N<sub>2</sub>) or 4% O<sub>2</sub> (4% O<sub>2</sub>, 5% CO<sub>2</sub> and balanced with N<sub>2</sub>) for 4 min at a rate of 20 L/min, then sealed and placed at 37°C.

**Adenovirus infection.** Adenoviral MnSOD, made originally by Dr. John Engelhardt (Zwacka et al., 1998), was manufactured at The University of Iowa's Vector Core Facility. For western and activity gel analyses, MCF-7 cells were plated into 60 mm dishes at a density of  $5 \times 10^5$  cells/plate in full media. The next day adenoviral *MnSOD* (prepared in 3% sucrose/PBS) was added to the cells containing 2 mL of media in each dish. To the appropriate dishes, 1.25 - 200 multiplicity of infection (MOI) of adenoviral constructs were added. The MOI for all experiments was calculated from the plaque forming units, which was determined by the Vector Core. Following a 24 h incubation, the viral particles were removed and fresh medium was added. All infections were done at 21% O<sub>2</sub>. Exposure to 1% O<sub>2</sub> and 4% O<sub>2</sub> was done at 24 hours after adenoviral infection.

**Cell homogenization for SOD activity gel and SOD western blot.** Cells were washed in phosphate-buffered saline (PBS: KCl 2.7 mM, KH<sub>2</sub>PO<sub>4</sub> 1.5 mM, NaHPO<sub>4</sub> 8 mM and NaCl 136.9 mM, pH 7.0), scrape harvested, and pelleted at 12,000 x g for 10 s in 1.5 mL microfuge tubes. The supernatant was removed, cells were resuspended in 50 mM phosphate buffer (pH 7.8) and sonicated on ice for 3 x 30 s using a Vibra Cell cup horn sonicator (Sonics and Materials, Inc.,



Danbury, CT) at maximum power. Protein concentration was estimated by the Bradford method (Biorad Laboratories, Hercules, CA) and standardized with bovine serum albumin.

**SOD activity gel.** In this technique, non-dissociating electrophoresis gels are run essentially by the method of Davis (Tulchin et al., 1976) with ammonium persulfate used as the initiator in the running gel (12.5%) and riboflavin-light in the stacking gel (5%). 120 µg total protein was used for activity analysis. Once run, the gels are stained for SOD activity by the method of Beauchamp and Fridovich (Beauchamp & Fridovich, 1971).

**SOD western blot.** The amount of immunoreactive MnSOD protein was measured by western blotting (Oberley et al., 1989). Briefly, cell homogenate (30 µg) were exposed to SDS-PAGE and transferred to PVDF (Millipore Corporation, Bedford, MA) membrane. The membrane was then probed with Rabbit anti-MnSOD IgG (1: 1000 dilution). The secondary antibody was goat anti-Rabbit IgG (Pharmingen/Transduction Laboratories, San Diego, CA) used at 1:5000 dilution. Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL) and exposed to film. The bands were examined with a computerized digital imaging system using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA). The IDV (integrated density values) were obtained by integrating all of the pixel values in the area of one band after correction for background.

**Protein harvest for HIF-1 $\alpha$  western blot.** Medium was removed from tissue culture dishes. After rinsing twice with PBS, 100 µL boiling lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris pH 7.4) was added to the cells. Cell lysates were then scraped and transferred into micro-centrifuge tubes and boiled for 5 min. Protein concentrations were determined with Bio-Rad DC protein assay (BioRad Laboratories, Hercules, CA) as described by the manufacturer.

**HIF-1 $\alpha$  western blot.** 30  $\mu$ g total protein was separated on a 4-20% gradient Tris-HCl polyacrylamide ready gel (BioRad Laboratories, Hercules, CA). Then the protein was electrotransferred onto a PVDF membrane (Millipore Corporation, Bedford, MA) by running at 100 V for 1 h. For HIF-1 $\alpha$  western blot, the primary antibody was mouse anti-HIF-1 $\alpha$  IgG (Pharmingen/Transduction Laboratories, San Diego, CA) used at 1:2000 dilution. For  $\alpha$ -tubulin western blot, the primary antibody was mouse anti-human  $\alpha$ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:5000 dilution. The secondary antibody used against both primary antibodies was goat anti-mouse IgG (Pharmingen/Transduction Laboratories, San Diego, CA) used at 1:2000 dilution. Blots were visualized using Chemiluminescent Developer (Pierce, Rockford, IL) and exposed to film. The bands were examined with a computerized digital imaging system using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA). The IDV (integrated density values) were obtained by integrating all of the pixel values in the area of one band after correction for background.

**Semiquantitative RT-PCR analysis.** Total RNA was extracted by Qiagen RNeasy kit (Qiagen Inc., Valencia, CA) as recommended by the manufacturer. The RT-PCR was carried out using a Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation. The RT-PCR product was electrophoresed on a 1% agarose gel.

**Statistics.** Pearson's regression and correlation analysis were used to determine the relationship between the MnSOD activities and HIF-1 $\alpha$  protein levels. The  $R^2$  and p-value was calculated using Microsoft Excel software.

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**Abbreviations:** GLUT-1: glucose transporter-1; HIF-1: hypoxia inducible factor-1; MnSOD: manganese superoxide dismutase; MOI: multiplicity of infection; ROS: reactive oxygen species; VEGF: vascular endothelial growth factor; VHL: von Hippel-Lindau.

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### Legend to Figure 1

Plasmid transfection of MnSOD suppressed HIF-1 $\alpha$  protein accumulation with 4 h exposure to 1% O<sub>2</sub>.

**a.** Western blot showing HIF-1 $\alpha$  protein levels in 12 different MnSOD stably transfected clones plus parental MCF-7 cells after 4 h exposure to 1% O<sub>2</sub>. The first 3 lanes were samples from: MCF-7 parental cells, Neo vector control, and a clone with the same activity as parental cells. The remaining 10 lanes were samples from clones with increasing MnSOD activity. The bottom row of numbers indicates the relative MnSOD activity compared to the parental MCF-7 cells. **b.**

Regression analysis shows a biphasic effect. HIF-1 $\alpha$  protein levels were quantified by densitometry and normalized to  $\alpha$ -tubulin levels. Similar results were obtained in a repeated experiment.



### Legend to Figure 2

Adenoviral transduction of MnSOD suppressed HIF-1 $\alpha$  protein accumulation with 4 h exposure to 1% O<sub>2</sub>. **a.** MnSOD activity gel analysis showed that MnSOD activity increased with increasing MOI (from 1.25 up to 200). Adenoviral transduction was performed at 21% O<sub>2</sub> and the transduced cells were exposed to 1% O<sub>2</sub> for 4 h. **b.** Western blots showed HIF-1 $\alpha$  protein levels in Ad*MnSOD* transduced cells exposed to 1% O<sub>2</sub> for 4 h.  $\alpha$ -Tubulin was used as an internal control. Similar results were obtained in a repeated experiment.

### Legend to Figure 3

Elevated MnSOD activities suppressed HIF-1 $\alpha$  protein accumulation at 4% O<sub>2</sub>. **a.** Plasmid transfection of MnSOD suppressed hypoxic (4% O<sub>2</sub>, 4 h) accumulation of HIF-1 $\alpha$  protein. Western blot shows HIF-1 $\alpha$  protein levels in different MnSOD stably transfected clones of MCF-7 cells with 4 h of exposure to 4% O<sub>2</sub>. The top row of numbers indicates the relative MnSOD activity in 12 different clones compared to MCF-7 WT cells.  $\alpha$ -Tubulin was used as an internal control. The 12 different clones are the same as used in **Figure 1**. Similar results were obtained in repeated experiments. **b.** Adenoviral transduction of MnSOD suppressed HIF-1 $\alpha$  protein accumulation at 4% O<sub>2</sub>. The top panel shows that MnSOD activity increased with increasing MOI (from 1.25 up to 200). Adenoviral transduction was performed at 21% O<sub>2</sub> and the transduced cells were exposed to 4% O<sub>2</sub>. The second and third panels shows western blots for HIF-1 $\alpha$  and  $\alpha$ -tubulin, respectively, from cells exposed to 4% O<sub>2</sub> for 4 h. Similar results were obtained in repeat experiments.

#### **Legend to Figure 4**

Steady-state levels of VEGF mRNA increased with time when MCF-7 WT cells were subjected to hypoxia. Cells were exposed to hypoxia (1% O<sub>2</sub>) for various times. Total RNA was then isolated and semiquantitative RT-PCR was carried out.  $\beta$ -Actin was used as the internal control. Similar results were observed in repeated experiments. M: DNA marker; N: negative control (without mRNA samples); 0, 2, 4, 6, 12, 24 h are different times of hypoxia exposure (1% O<sub>2</sub>).

### Legend to Figure 5

MnSOD overexpression suppressed hypoxic induction of VEGF mRNA in MCF-7 cells. **a.** A representative result shows that MnSOD overexpression suppressed hypoxic (1% O<sub>2</sub>, 6 h) induction of VEGF mRNA in 7 distinct MnSOD stably transfected clones as well as parental MCF-7 cells. M: DNA marker; N: negative control (without mRNA samples). **b.** Densitometric analysis of the RT-PCR results. Values represent mean  $\pm$  S.E.M. of three independent experiments.  $\beta$ -Actin was used as the internal control. **c.** Adenoviral transduction of MnSOD suppressed hypoxic induction of VEGF. Lanes 3 & 6 were samples from MCF-7 WT cells; lanes 4 & 7 were samples from MCF-7 cells transduced with 50 MOI of LacZ; lanes 5 & 8 were samples from MCF-7 cells transduced with 50 MOI of MnSOD. Lanes 3-5 were samples from cells without hypoxic exposure (21% O<sub>2</sub>); lanes 6-8 were samples from cells with hypoxic exposure (1% O<sub>2</sub>) for 6 h. M: DNA marker, the bright band is molecules with 600 bp; N: negative control (without mRNA samples); hypoxia (-): 21% O<sub>2</sub>; hypoxia (+): 1% O<sub>2</sub>. Similar results were observed with a repeated experiment.

### Legend to Figure 6

MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. **a.** Hypoxia induced the secretion of VEGF protein in a time-dependent manner in MCF-7 WT cells. MCF-7 WT cells were exposed to hypoxia (1% O<sub>2</sub>) for various times. VEGF concentration was measured with a Human VEGF-ELISA kit (R&D Systems, Minneapolis, MN). VEGF production increased when cells were exposed to hypoxia for longer times. VEGF was mainly secreted outside the cell as the concentration of VEGF in tissue culture medium was much higher than that in cell lysate. Values represent mean  $\pm$  S.D of three measurements of a single sample. **b.** MnSOD overexpression suppressed VEGF protein secretion in MCF-7 cells. VEGF protein was measured after 12 h of hypoxia (1% O<sub>2</sub>) in 7 distinct clones plus MCF-7 parental cells with various MnSOD activities. Values represent mean  $\pm$  S.E.M. of three independent experiments.

### **Legend to Figure 7**

VEGF secretion over time was different in an MCF-7 clone with intermediate MnSOD activity compared to a clone with high MnSOD activity. SOD50 has a 3-fold increase in MnSOD activity and Mn11 has a 19-fold increase in MnSOD activity compared to parental cells. VEGF protein was greatly induced in WT cells relative to MnSOD-overexpressing clones. Values represent mean  $\pm$  S.E.M. of three independent experiments.

Figure 1

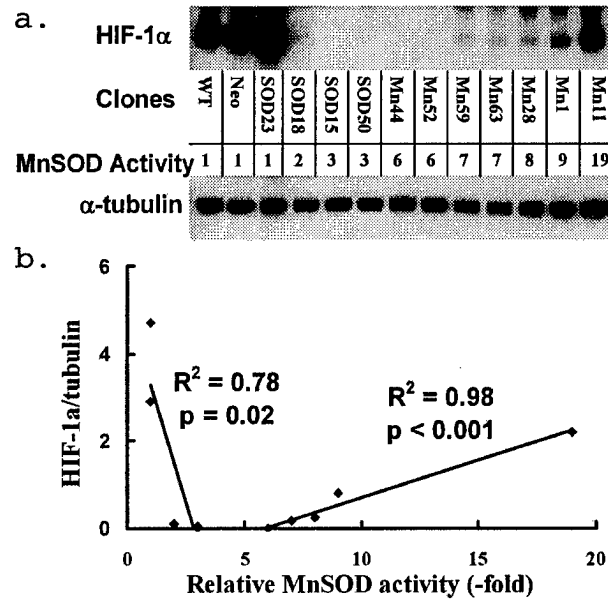


Figure 2

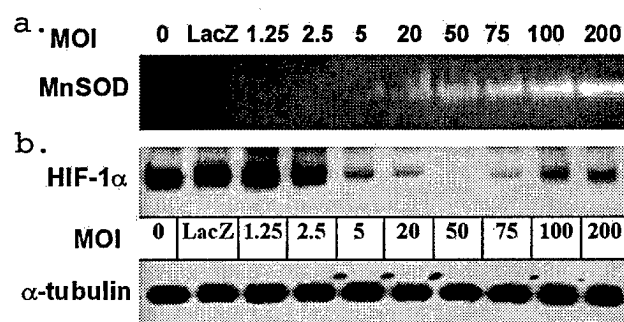




Figure 3

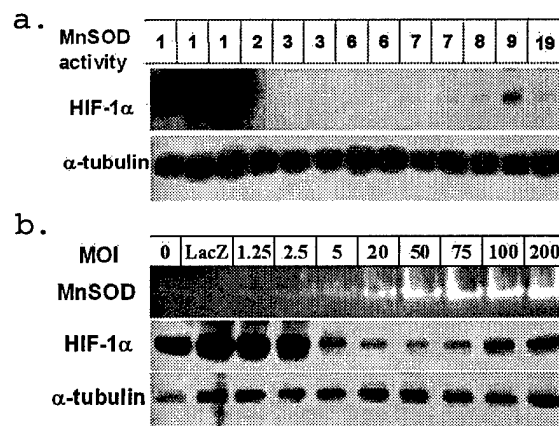


Figure 4

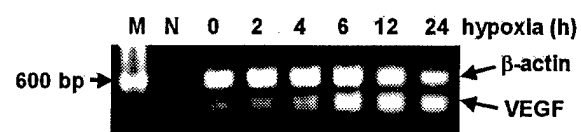


Figure 5

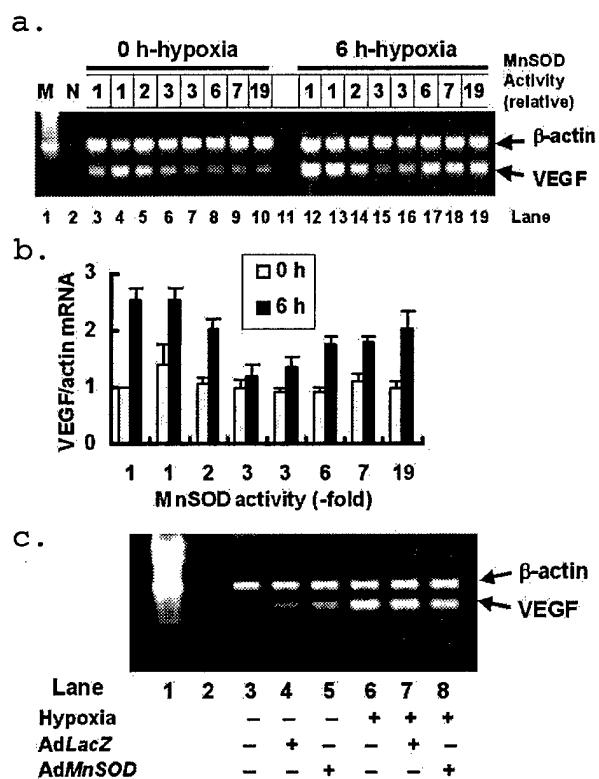


Figure 6

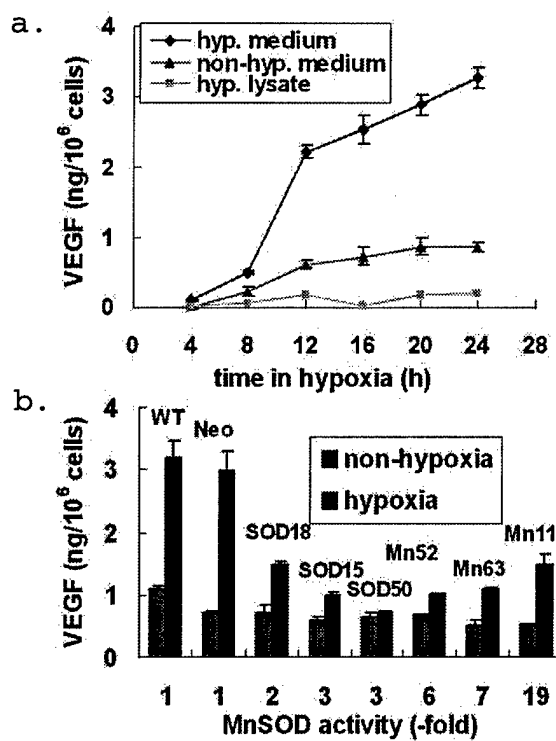


Figure 7

